(51) International Patent Classification 6 :	A1
C12N 15/12, C07K 14/82, 14/47, G01N 33/574, A61K 38/17, 38/04	

(54) Title: INTERRUPTION OF BINDING OF MDM2 AND p53 PROTEIN AND THERAPEUTIC APPLICATION THEREOF

(57) Abstract

Compounds for use in interfering with the binding between p53 and MDM2 or a protein having a p53 binding site analogous to that of MDM2, the compound being selected from the group consisting of a peptide having up to twenty-eight amino acids which is able to disrupt or prevent binding between p53 and MDM2, or a functional peptide analogue thereof. Methods for detecting such compounds and their application in the diagnosis and treatment of tumours is also described and claimed.

INTERRUPTION OF BINDING OF MDM2 AND P53 PROTEIN AND THERAPEUTIC APPLICATION THEREOF

5   The invention relates to the area of cancer detection and
       therapeutics. More particularly it relates to the
       prevention or disruption of the inactivation of the p53
       tumour suppressor which occurs as a result of the binding
       of a protein through the amino acid motif within the
10   region of p53 represented by amino acids 16-30
       QETFSDLWKLLPENN (SEQ ID NO:1) of the human p53 protein.
       An example of such a protein is the oncogene protein MDM2
       (human MDM2).

15

       Inactivation of the p53 tumour suppressor is a frequent
       event in human neoplasia. The inactivation can occur by
       mutation of the p53 gene or through binding to viral or
       cellular oncogene proteins, such as the SV40 large T
20   antigen and MDM2. While the mechanism through which
       wild-type p53 suppresses tumour cell growth is as yet
       poorly defined it is clear that one key feature of the
       growth suppression is the property of p53 to act as a
       transcription factor (Farmer, G., et al. (1992). Nature,
25   358, 83-86; Funk, W.D. et al. (1992). Mol. Cell. Biol.,
       12, 2866-2871; Kern, S.E., et al. (1992). Science, 256,
       827-830). Currently, considerable effort is being made to
       identify growth control genes that are regulated by p53
       binding to sequence elements near or within these genes.
30   A number of such genes have been identified. In cases
       such as the muscle creatine kinase gene (Weintraub, H.,
       et al. (1991). Proc. Natl. Acad. Sci. U.S.A., 88, 4570-
       4571; Zambetti, G.P., et al. (1992). Genes Dev., 6, 1143-
       1152) and a GLN retroviral element (Zauberman, A., et
35   al. (1993). Embo J., 12, 2799-2808) the role these genes
       might play in the suppression of growth control is
       unclear. Yet there are other examples, namely *mdm2*

2

(Barak, Y., et al. (1993). Embo J., 12, 461-468; Wu, X., et al. (1993). Genes Dev., 7, 1126-1132) GADD 45 (Kastan, M.B., et al. (1992). Cell, 71, 587-597) and WAF1 or CIP1 (El-Beiry, W.S., et al. (1993). Cell, 75, 817-825;

5   Harper, J.W., et al. (1993). Cell., 75, 805-816) where their involvement in the regulation of cell growth is better understood.

In the present text "*mdm2*" refers to the oncogene and

10   "MDM2" refers to the protein obtained as a result of expression of that gene.

Mdm2, a known oncogene, was originally found on *m*ouse *d*ouble *m*inute chromosomes (Cahilly-Snyder., L., et al.

15   (1987) Somatic Cell Mol. Genet. 13, 235-244). Its protein product was subsequently found to form a complex with p53, which was first observed in a rat fibroblast cell line (Clone 6) previously transfected with a temperature sensitive mouse p53 gene (Michalovitz, D., et al. (1990).

20   Cell, 62, 671-680). The rat cell line grew well at 37°C but exhibited a G1 arrest when shifted down to 32°C, which was entirely consistent with an observed temperature dependent switch in p53 conformation and activity. However, the p53-MDM2 complex was only

25   observed in abundance at 32°C, at which temperature p53 was predominantly in a functional or "wild-type" form (Barak, Y. et al. (1992). Embo J., 11, 2115-2121 and Oren, 1992; Momand, J., et al. (1992). Cell, 69, 1237-1245). By shifting the rat cell line down to 32°C and

30   blocking de novo protein synthesis it was shown that only "wild-type" p53 induced expression of the *mdm2* gene, thereby accounting for the differential abundance of the complex in terms of p53 transcriptional activity (Barak, Y., et al. (1993). Embo J., 12, 461-468)

35   The explanation was further developed by the identification of a DNA binding site for wild-type p53 within the first intron of the *mdm2* gene (Wu, X., et al.

(1993). Genes Dev., 7, 1126-1132). Reporter constructs
employing this p53 DNA binding site revealed that they
were inactivated when wild-type p53 was co-expressed with
MDM2.

5

This inhibition of the transcriptional activity of p53
may be caused by MDM2 blocking the activation domain of
p53 and/ or the DNA binding site. Consequently, it was
proposed that mdm2 expression is autoregulated, via the
10   inhibitory effect of MDM2 protein on the transcriptional
activity of wild-type p53. This p53-mdm2 autoregulatory
feedback loop provided a novel insight as to how cell
growth might be regulated by p53. Up to a third of human
sarcomas are considered to overcome p53-regulated growth
15   control by amplification of the mdm2 gene (Oliner, J.D.,
et al. (1992). Nature, 358, 80-83). Hence the
interaction between p53 and MDM2 represents a key
potential therapeutic target.

20   The cDNA sequence encoding the human MDM2 protein (which
is also referred to as "HDM2" in the art) is known from
WO93/20238. This application also discloses that human
MDM2 protein binds with human p53 and it has been
suggested that molecules which inhibit the binding of
25   MDM2 to p53 would be therapeutic by alleviating the
sequestration of p53. However it is also suggested that
the p53 and MDM2 binding site is extensive, including
amino acid residues 13-41 of p53 as well an additional
nine to thirteen residues at either the amino or carboxyl
30   terminal side of the peptide are also involved. This
would indicate that a large polypeptide or other large
molecule would be required in order to significantly
interfere with the binding.

35   The applicants have therefore sought to immunochemically
characterize the p53-MDM2 complex, and also determine in
fine detail the MDM2 binding site on p53.

4

Surprisingly, it has been found that only a relatively small number of amino acids within the p53 protein are involved in binding to MDM2.

5

The precise identification of this binding site is vital to allow the rational design of molecules which will disrupt or prevent binding between p53 and MDM2 or proteins containing analogous p53 binding sites. In 10 addition it allows for the design of screening procedures which will enable compounds which can disrupt or prevent the binding interaction to be accurately and rapidly identified.

15 The applicants have found that the site on the p53 protein which is responsible for binding to MDM2 is a small sequence of only six amino acids, of which three amino acids have been found to be critical. This sequence is represented by the sequence TFSDLW (SEQ ID 20 NO:2) in human (amino acids 18-23 in the sequence) and TFSGLW (SEQ ID NO:4)(amino acids 18-23) in mouse, of which the critical amino acids appear to be F--LW (SEQ ID NO:4). By disrupting or preventing p53 from binding in this specific region, the deleterious effects of binding 25 to MDM2 or proteins having an analogous p53 binding site can be avoided. Proteins having a p53 binding site which is analogous to that of MDM2 will generally comprise oncogene proteins which bind to p53 through the amino acid motif within the region of p53 represented by amino 30 acids 16-30 (QETFSDLWKLLPENN) (SEQ ID NO:1) of the human p53 protein.

This finding has recently been reinforced by a report that two components of the transcriptional machinery, 35 namely TAFII40 and TAFII60, require Leu-22 and Trp-23 for them to bind to p53 and mediate p53 transcriptional activity (Thut- CJ, et al., 1995, Science 267:100-4).

The same two amino acids of p53 are critical for the
binding of MDM2 as disclosed above as well as Ad E1b,
thus strengthening the hypothesis that MDM2 and E1b act
by binding to p53 and blocking the transcriptional
5   activity of p53.

Hence the present invention provides a method for
interfering with the binding between p53 and MDM2 or an
oncogene protein having an analogous p53 binding site,
10   which method comprises administering a effective amount
of a compound, selected from the group consisting of a
peptide having up to twenty eight amino acids which is
able to disrupt or prevent the binding between p53 and
MDM2, or a functional peptide analogue thereof.
15

It may be expected that small peptides, for example of
from 4 to 10 amino acids, suitably from five to 10 amino
acids, or peptide analogues thereof would be particularly
suitable in such a process. Peptides which would be of
20   particular interest are those which show a consensus with
the fragment of p53 which has been found to be crucial
for binding. Such peptides include fragments of p53
protein which includes at least some of amino acids 18-23
within the sequence of human p53, as identified in
25   WO93/20238 or a peptide analogue thereof. Suitably these
peptides are those which are circular, linear or
derivatised to achieve better penetration of membranes.

Novel peptides or peptide analogues of this type form a
30   further aspect of the invention.

Hence preferred peptides include the sequence FxxLW (SEQ
ID NO:4) such as TFSDLW (SEQ ID NO:2) or a portion
thereof. As used herein, 'x' refers to any amino acid.
35   In a preferred embodiment, an aspartate residue in the
sequence is replaced by a glutamate residue so that the
sequence is FxELW (SEQ ID NO:5 such as TFSELW (SEQ ID

6

NO:6.

Other compounds which may interfere with the binding
include organic compounds which are modelled to achieve
the same three dimensional structure as the said region
of the p53 peptide. Hence in an alternative embodiment
the invention provides an organic compound which is
modelled to resemble the three dimensional structure of
the amino acids represented by the sequence F--LW (SEQ ID
NO:4) as it appears in human p53 in the region of amino
acids 19-23 and which binds to human MDM2.   In
particular the organic compound may be modelled to
resemble the three dimensional structure of the sequence
TFSDLW (SEQ ID NO:2) as it appears in the region of amino
acides 18-23 of human p53.

A suitable oncogene protein is MDM2 but the disruption of
binding of p53 to other oncogene proteins containing a
p53 binding site analogous to that of MDM2 are included
within the scope of the present invention. Examples of
other such oncogene proteins include the adenovirus EIB
58kD protein, the Tata box binding protein TBP and the
transcription factor of the E2F family.

As used herein the expression 'peptide analogue' refers
to peptide variants or organic compounds having the same
functional activity as the peptide in question, in
particular which interfere with the binding between p53
and MDM2. Examples of such analogues will include
chemical compounds which are modelled to resemble the
three dimensional structure of the sequence TFSDLW, (SEQ
ID NO:2) and in particular the arrangement of the F--LW
(SEQ ID NO:4) amino acids as they appear in human p53,
which compounds bind to human MDM2.

Suitable modelling techniques are known in the art. This
includes the design of so-called 'mimetics' which

involves the study of the functional interactions of the
molecules and the design of compounds which contain
functional groups arranged in such a manner that they
could reproduce those interactions.

5

The designing of mimetics to a known pharmaceutically
active compound is a known approach to the development of
pharmaceuticals based on a "lead" compound. This might
be desirable where the active compound is difficult or

10   expensive to synthesise or where it is unsuitable for a
particular method of administration, eg peptides are
unsuitable active agents for oral compositions as they
tend to be quickly degraded by proteases in the
alimentary canal. Mimetic design, synthesis and testing

15   is generally used to avoid randomly screening large
number of molecules for a target property.

There are several steps commonly taken in the design of a
mimetic from a compound having a given target property.

20   Firstly, the particular parts of the compound that are
critical and/or important in determining the target
property are determined. In the case of a peptide, this
can be done by systematically varying the amino acid
residues in the peptide, eg by substituting each residue

25   in turn. These parts or residues constituting the active
region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is
modelled to according its physical properties, eg

30   stereochemistry, bonding, size and/or charge, using data
from a range of sources, eg spectroscopic techniques, X-
ray diffraction data and NMR. Computational analysis,
similarity mapping (which models the charge and/or volume
of a pharmacophore, rather than the bonding between

35   atoms) and other techniques can be used in this modelling
process.
In a variant of this approach, the three-dimensional

8

structure of the ligand and its binding partner are
modelled. This can be especially useful where the ligand
and/or binding partner change conformation on binding,
allowing the model to take account of this the design of
the mimetic.

A template molecule is then selected onto which chemical
groups which mimic the pharmacophore can be grafted. The
template molecule and the chemical groups grafted on to
it can conveniently be selected so that the mimetic is
easy to synthesise, is likely to be pharmacologically
acceptable, and does not degrade *in vivo*, while retaining
the biological activity of the lead compound. The
mimetic or mimetics found by this approach can then be
screened to see whether they have the target property, or
to what extent they exhibit it. Further optimisation or
modification can then be carried out to arrive at one or
more final mimetics for *in vivo* or clinical testing.

In order to identify compounds which are useful in the
above described methods, compounds may be screened for
interference of the MDM2/p53 interaction. Suitably
screening methods would be based upon observations with
regard to compounds which interfere with the binding
between peptides which comprise or represent the binding
site of an oncogene protein such as MDM2 and p53, based
upon the information regarding said binding site given
herein. Such methods include immunoassay techniques such
as radioimmunoassay (RIA) and enzyme linked
immunoabsorbent assay (ELISA) which are well known in the
art. Particularly suitable techniques are competitive
assay techniques where a peptide or reagent which either
is or represents one of the peptides or reagents which
either is or represents either p53 or the oncogene
protein is exposed to a compound under test and the other
one of the peptides or reagents which either is or
represents either p53 or the oncogene protein. The

9

prese :e of bound complex is then detected. This may be
achieved either by labelling of the said one of said
peptides or reagent for example using a gold or other
visible label, or by administering a labelled antibody or
5   sequence of antibodies, one of which includes a label, in
a conventional manner. Suitable antibodies for example
for MDM2 and p53 are described herein. Suitably one of
the peptides or reagents representing p53 or the oncogene
protein is immobilised on a support.
10

Hence the invention further provides a method of
identifying compounds which interfere with the binding of
human MDM2 to human p53,said me⁻hod comprising
        immobilising a first peptice molecule,
15   adding a compound to be tested and a second peptide
molecule; detecting the presence of bound second peptide
at the immobilisation site;
wherein one of the first peptide or second peptide is
MDM2 or an oncogene protein having a p53 binding site
20   analogous to that of MDM2 or a fragment thereof which
includes said binding site, and the other is a fragment
of human p53 of from five to twenty eight amino acids
including the amino acid residues FxxLW (SEQ ID NO:4) or
a peptide analogue thereof.
25

A immunoassay for detecting binding is illustrated
hereinafter.

A biotinylated peptide containing the MDM2 binding site
30   fo p53 from amino acids 16-25 (or smaller peptides as
described above containing for example TFSDLW) (SEQ ID
NO:2) would be immobilised on a streptavidin coated ELISA
plate. Recombinant MDM2 protein would be added to these
ELISA plates in the presence of absence of test compounds
35   or agents. This would then be incubated for 2 hours at
4°C. Bound MDM2 would be detected by a standard ELISA
procedure. The inhibitory or stimulatory effect of these

reagents would be determined by reference to control
wells in which no such test compounds were included.
Further experimental details are as described hereinafter
and the binding assay is illustrated in Figure 7.

The invention includes quantitative assays. For example
there is provided a method of identifying compounds which
interfere with the binding of human MDM2 to human p53,
said method comprising binding a predetermined quantity
of a first peptide which is detectably labelled to a
peptide, adding a compound to be tested; and determining
the quantity of the first protein which is displaced from
or prevented from binding to the second peptide, wherein
one of the first peptide or the second peptide is MDM2 or
a peptide having a p53 binding site analogous to that of
MDM2, and the other is a fragment of human p53 of from
six to twenty eight amino acids including the amino acid
residues 18-23 in the sequence of human p53 as set out in
WO93/20238, or a peptide analogue thereof.

Assays which include fragments of p53 including the MDM2
binding site as characterised above, or peptide analogues
thereof form a further part of the invention. The assay
may be formulated as a kit which also forms part of the
invention. A particularly useful form of the assay would
be one which was adapted to test levels of oncogene
protein in biological samples. Such a kit would comprise
a fragment of p53 or a peptide analogue as binding agent
thereof together with an antibody which is specific for
the oncogene protein such as MDM2. Alternatively
antibodie(s) able to detect bound complex may be included
in the kit.

This could be used in diagnosis to measure the levels of
oncogene protein or MDM2 in blood samples in the case of
leukaemias or solid carcinomas such as sarcomas and
glioblastomas.

11

Suitably in the above described assay methods, the
oncogene protein is human MDM2 and the other protein
comprises a fragment of human p53 of from 12 to 28 amino
acids including the sequence TFSDLW (SEQ ID NO:2).

5

The methods can be readily adapted to provide a high
throughput screen, for example by carrying out the
process in a 96-well format. Automated screening
techniques can be applied in these circumstances as would

10   be understood in the art. Compounds from various sources
can be screened in large numbers. One potential source
of compounds are the available synthetic combinatorial
peptide libraries.

15   The use of compounds identified by this screening method
in the treatment of tumours forms a further aspect of the
invention.

Methods of treatment of conditions such as cancer and

20   other malignancies are envisaged by the administration of
the compounds of the invention.

Hence the invention also provides a method for inhibiting
the growth of tumour cells which contain a human MDM2

25   gene amplification which method comprises administering a
effective amount of a compound which interferes with the
binding between p53 and an MDM2, said compound being
selected from the group consisting of a peptide having up
to twenty eight amino acids which is able to disrupt or

30   prevent the binding between p53 and MDM2, or a functional
peptide analogue thereof.

Preferably in the above-described method of treatment,
the compound is a peptide of from six to twenty eight

35   amino acids which has a consensus with a region of human
p53 and includes the sequence FxxLW (SEQ ID NO:4) for
example TFSDLW (SEQ ID NO:2).

12

Alternatively, the compound used in the method is peptide
analogue such as an organic compound which binds to the
same site on MDM2 as the sequence TFSDLW (SEQ ID NO:2).

5   For use in these applications, the compounds are suitably
applied in the form of compositions with pharmaceutically
acceptable carriers. These may be solid or liquid for
carriers and the compositions suitable for oral or
parenteral application as would be understood in the art.
10   Dosages of the compounds will depend upon the patient,
the particular condition and the nature of the specific
compound chosen. For example, when the compound is a
peptide fragment dosages of from 0.1 to 10 mg/Kg may be
effective.

15

It has been suggested (Picksley and Lane. (1993).
Bioessays. 15, 10, 689-690) that mdm2 expression is
autoregulated in a feedback loop, via the inhibitory
effect of MDM2 protein on the transcriptional activity of
20   wild-type p53. Any interference with the binding between
p53 and MDM2 in accordance with the present invention,
will affect the p53-MDM2 autoregulatory loop. Given
p53's role as guardian of the genome, compounds which
have such an effect could enhance the activity of other
25   therapeutic agents.

Hence in a further aspect the invention comprises a
pharmaceutical composition comprising synergistic amount
of a compound of the invention in combination with
30   another anticancer therapeutic agent.

DNA encoding an MDM2-binding, p53 derived peptide, or
multiple copies thereof may also be administered to
tumour cells as a mode of administering the peptide.
35   Hence the invention provides a method for inhibiting the
growth of tumour cells which contain a human MDM2 gene
amplification, the method comprising applying to said

13

tumour cells a DNA molecule which expresses a polypeptide
comprising a portion of p53 or a variant thereof, said
portion comprising amino acids 18-23 of p53, said
polypeptide being capable of binding to human MDM2.

5

The DNA will typically be in an expression construct,
such as a retrovirus, DNA virus, or plasmid-vector, which
has the DNA elements necessary for expression properly
positioned to achieve expression of MDM2-binding peptide.

10   The DNA can be administered *inter alia* encapsulated in
liposomes, or in any other form known to the art to
achieve efficient uptake by cells.

By identifying the binding site so specifically, the

15   applicants have opened up the possibility of making small
therapeutic compounds which will target this site
specifically. This is advantageous since small molecules
are more likely to be able to penetrate into a cell and
hence be therapeutically active. Furthermore the

20   diagnostic process can be effected more accurately and
using simpler molecules as a result of this discovery.

The invention will be described further by reference to
the accompanying drawings in which: Figures 1A-1B show

25   Western blots of immunoprecipitates of MDM2, p53 and the
MDM2-p53 complex obtained from Clone 6 cells.

Figures 2A-2B are graphs showing the results of two-site
immunoassays to determine the levels of MDM2, p53 and

30   MDM2-p53 complex in Clone 6 cells grown at 32°C for 24
hours (A) or continuously at 37°C (B).

Figures 3A-3C are graphs showing the results of binding
MDM2 to a peptide library as determined by ELISA assay

35   using monoclonal antibody 4B2. The library of human and
mouse p53 had been challenged with insect cell extract
alone (SF9) and insect cell extract expressing mouse MDM2

14

(SF9 Mus MDM2. The results of peptides numbers 3-50 of
the N-terminal to mid region of human p53 are shown in
3A, and the remainder of the human p53 amino acid
sequence and the N-terminal sequence of mouse p53 in 3B.
5   3C shows the results from a control experiment using
certain peptides from B in order to verify the
specificity of the detecting antibody 4B2.

Figure 4 identifies the peptide sequences to which MDM2
10   bound , and defines the consensus binding site on human
and mouse p53;

Figures 5A-5C show the key residues required for binding
MDM2, antibody DO-1 and antibody Bp53-19 respectively;
15

Figure 6 shows the level of binding between MDM2 and a
series of peptides based upon the sequence of amino acids
18-23 of p53; and

20   Figure 7 illustrates an immunoassay procedure of the
invention.

The first indication of an interaction between MDM2
25   protein and p53 protein emerged from work on a rat cell
line, Clone 6, which expressed a temperature sensitive
mutant form of mouse p53 (Barak and Oren, 1992;
Michalovitz, D., et al. (1990). Cell, 62, 671-680;
Momand, J., et al. (1992). Cell, 69, 1237-1245). MDM2
30   was readily observed to form a complex at 32°C with p53
but was just detectable when cells were grown at 37°C

The formation of a p53-MDM2 complex in Clone 6 cells at
32°C and 37°C was re-examined in a quantitative manner.
35   The results confirm previous immunoprecipitation
observations that the level of MDM2 at the lower
temperature is significantly elevated, approximately 10-

15

30 fold greater than that at 37°C, at which temperature
MDM2 is only just detectable. Consequently, the p53-MDM2
complex is readily observed at 32°C and not at 37°C. The
levels of p53 also vary at the two different

5   temperatures. However, the p53 levels are elevated
approximately five fold at 37°C as compared with that at
32°C, -the opposite behaviour to that of MDM2.
Accordingly, the difference in the levels of p53 and MDM2
are likely to have alternative explanations. In the case

10   of MDM2 other groups have established that the increase
of MDM2 at 32°C is due to increased transcription of MDM2
due to a conformational change in p53 to a presumed
transcriptional active form (Barak, Y., et al. (1993).
Embo J., 12, 461-468; Wu, X., et al. (1993). Genes Dev.,

15   7, 1126-1132). The same explanation does not apply for
p53 even though wild type p53 is required for p53
expression (Deffie, A., et al. G. (1993). Mol. Cell.
Biol., 13, 3415-3423), and is probably explained by the
increased half life of the mutant conformation of p53 at

20   37°C (Gannon J.V. et al (1991) Nature, 349, 802-806).
Data described herein after using both direct observation
of the p53-MDM2 complex by ELISA and immunoprecipitation
combined with the indirect inference of the loss of the
Bp53-19 epitope suggested that nearly all p53 molecules

25   are complexed to excess MDM2 protein in C6 cells at 32°C.
This is not consistent with the powerful p53 dependant
transcriptional response seen in these cells at this
temperature and suggests that either that complexing to
MDM2 is unable to completely inactivate p53 in vivo or

30   that small amounts of "free" p53 may be very active. The
complex between p53 and MDM2 may be regulated in cells to
release functional p53 at the individual cell level
perhaps as a cell cycle dependant response.

35   The present invention is based upon the identification of
the minimal MDM2 binding site to be TFSD/GLW (SEQ ID
NOS:2 and 3). This site is in a location broadly

reported by other groups to be the MDM2 binding domain of
p53, specifically aal-41 and 13-57 (Oliner, J.D., et al.
(1993). Nature, 362, 857-860), aal-52 (Chen, J., et al.
(1993). Mol Cell Biol, 13, 4107-14) and aal-159 (Brown,
5   D.R., et al. (1993). Mol. Cell. Biol., 13, 6849-57.)
Notably, a construct generated by Oliner and co-workers
encompassing aa13-41 of p53 was not sufficient for MDM2
binding in a three hybrid protein system, and differs
from our observations. The disparity might be explained
10   by the close proximity of the fusion protein sequence
adjacent to the TFSDGLW (SEQ ID NO:7) sequence at aa18-23
as the present data does show that flanking sequences do
contribute in a minor way to MDM2 binding. The TFSD/GLW
(SEQ ID NOS:2 and 3) sequence is very closely adjacent to
15   the transactivation domain aa20-42 (Unger, T., et al.
(1992). Embo J., 11, 1383-1390), and as shown by others
the binding of MDM2 to this site interferes with the
transcriptional activity of p53 (Oliner, J.D., et al.
(1993). Nature, 362, 857-860). While substitution
20   analysis of the MDM2 binding site on p53 identified the
TFSD/GLW (SEQ ID NOS:2 and 3) sequence to be the key
region required for MDM2 to bind p53, other residues
flanking this site also contribute in a minor way to MDM2
binding, but clearly the TFSD/GLW (SEQ ID NOS: 2 and 3)
25   sequence is a minimal target for agents that might
disrupt complex formation without effecting the
transactivation activity (for which as yet the key
residues are undetermined). The first two residues TF
are part of the conserved box I, and the latter four
30   SD/GLW (SEQ ID NOS:8 and 9) are outside but are also part
of a region of p53 that is conserved from Xenopus to man.

The corresponding binding site on MDM2 for p53 has
35   variously been reported to be between aal-121, 19-102
(Chen, J., et al. (1993). Mol Cell Biol, 13, 4107-14)
together with aa102-294 or 249-491, and also 1-221

17

(Brown, D.R., et al. (1993). Mol. Cell. Biol., 13, 6849-57). Notably, a monoclonal antibody against the N-terminal region of human MDM2, 3G5 (maps at aa59-89) is able to immunoprecipitate MDM2 but not co-

5   immunoprecipitate p53 (Chen, J., et al. (1993). Mol Cell Biol, 13, 4107-14), an analogous observation to our findings with antibody Bp53-19.

     The binding of MDM2 to p53 peptides has obvious parallels
10   to a similar study that used small peptides to identify the binding sites of Adenovirus E1A and human papilloma virus E7 for a range of proteins including retino-blastoma protein, p107, cyclin A and p130 (Dyson, N., et al. (1992a). J. Virol., 66, 4606-4611). The MDM2 binding
15   site on p53, appears to be a single domain rather than two domains as in the case of E1A and E7. The MDM2 binding site on p53 overlaps precisely with a highly immunogenic epitope on the protein; many independently isolated monoclonal antibodies to p53 recognise the site,
20   and antibodies to it are present in the sera of cancer patients (Schlichtholtz, B., et al. (1993). Cancer Res., 52, 6380-6384). This suggests that it has an exposed and defined structure. It is possible that the amino acid sequence of the complementarity determining regions of
25   these antibodies will show homology to the p53 binding site of MDM2. It also suggests that anti-p53 antibodies used to examine p53 levels where high levels of MDM2 are present must be chosen with care. Binding of MDM2 to this site may be regulated by phosphorylation since there
30   is a DNA-dependent kinase site at serine 20 (Less-Miller, S.P. et al. (1990). Mol. Cell. Biol., 10, 6472-6481) and other phosphorylation sites at serine 6, 9 and 15 (Samad, A., et al. (1986). Proc. Natl. Acad. Sci. U.S.A., 83, 897-901; Meek, D.W. et al. (1988). Mol. Cell. Biol., 8,
35   461-465; Meek and Eckhardt, 1988).

     The following examples are provided to exemplify various

18

aspects of the invention and are not intended to limit the scope of the invention.

In these examples, the following materials and methods
5   were used.

Materials and methods
Cell culture
Clone 6 cells (Michalovitz et al., 1990) were grown in
10   Dulbecco's Modified Eagle Medium (DMEM) supplemented with
10% FCS at either 32 or 37°C. The Spododoptera
frugiperda cell line, SF9, was grown at 27°C in ExCell
400 medium (J.R. H. Biosciences, Sera-Lab, UK)
supplemented with 5% FCS and glutamine.

15

Expression of MDM2 in insect cells
The mouse mdm2 gene was obtained from a mouse prostate
cell line (Lu et al., 1992) by polymerase chain reaction
and then cloned into a Spododoptera frugiperda expression
20   vector pVL1393 using standard DNA and baculovirus
expression techniques. An expression clone was
identified by the production of a 90-95kDa protein that
was recognized by anti-MDM2 antibodies.

25   **Antibodies**
p53 protein was detected using the polyclonal sera CM1
(Midgley, C.A., et al. (1992). J. Cell. Sci., 101, 183-
189), or monoclonal antibodies PAb421 (Harlow E et al.,
(1981) J. Virol., 39, 861-869) and Bp53-19 (Bartek J., et
30   al (1993). J. Pathol., 169, 27-34). MDM2 was detected
using rabbit anti-MDM2 polyclonal sera (Barak, Y., et al.
(1993). Embo J., 12, 461-468) or monoclonal antibody 4B2
(Chen et al., 1993) and SMP14 (a previously unreported
monoclonal antibody raised by us against a peptide,
35   CSRPSTSSRRRAISE,(SEQ ID NO:10) containing part of the
human MDM2 sequence from aa154 to 167 (Oliner, J.D., et
al. (1992). Nature, 358, 80-83) the first cysteine is not

part of the MDM2 sequence but was added to provide an
extra coupling option). An antibody, PAb419, raised
against SV40 large T antigen (Harlow, E., et al. (1981).
J. Virol., 39, 861-869) was used as an irrelevant control
5   for immunoprecipitations.

Immunoprecipitation
Cells were lysed in ice-cold NET buffer (50mM Tris-HCI,
pH8.0, 150mM NaCl, 5mM EDTA, 1% NP40) containing 1mM
10   phenylmethylsulphonyl fluoride, for 30 min at 4°C.
Debris was removed from the cell extract by
centrifugation at 14,000 rpm in a refridgerated Eppendorf
centrifuge. The immunoprecipitation procedure was
essentially as previously described (Gannon, J.V., et al.
15   (1990). Embo J., 9, 1595-1602) using 1μg of purified
mouse monoclonal antibody, and Protein G Sepharose beads
(Pharmacia) for both pre-absorption of the cell extracts
and subsequent isolation of the antibody-protein complex.

20   Screening of p53 peptide library

Peptide libraries of the entire human p53 protein and a
partial N-terminal region of the mouse p53 protein was
obtained from Chiron Mimotopes P/L (Victoria, Australia).
25   The libraries were in the form of 15 mer peptides linked
to biotin via an additional peptide spacer region of
serine-glycine-serine-glycine, and each peptide shared a
5 amino acid overlap with the previous peptide in the
primary sequence. ELISA plates were coated with 100μl of
30   5 μg/ml streptavidin (Vector labs) per well and incubated
overnight at 37°C and then blocked with phosphate
buffered saline (PBS) containing 2% bovine serum albumin
(BSA) for 1 hour at room temperature. The stock
biotinylated peptides were diluted to 5 μg/ml in PBS
35   containing 0.1% BSA and 50 μl of each were plated into
designed wells and then incubated at room temperature for
1 hour. The plates were washed four times with PBS

20

containing 0.1% Tween 20 before addition of the cell
extract (50 μl of 1-4 mg/ml per well) or purified
protein. The plates were incubated at 4°C for 2-3 hours,
before washing four times with PBS containing 0.1% Tween
5   20 to remove unbound protein. In the case of cell
extracts bound protein was detected with the appropriate
primary antibody at 1-3 μg/ml, and followed by an anti-
mouse horse radish peroxidase conjugate and 3'3'4'4'-
tetramethyl benzidine (TMB) substrate as in the standard
10   ELISA assay (Harlow, E., et al (1988). Antibodies: a
laboratory manual. New York. Cold Spring Harbor
Laboratory Press and Lane, 1988).

The levels of p53, MDM2, and complexes thereof were
15   determined by a two site immunoassay using stated
antibodies. Mouse monoclonal antibodies were used as the
solid phase by incubating Falcon microtitre dish wells
with 50 μl of a 30 μg/ml solution of purified antibody
overnight at 4°C. The plates were blocked with 2% bovine
20   serum albumin in PBS for 2 h at room temperature, and
washed with PBS. Cell extracts were prepared as
described for immunoprecipitations and then serially two-
fold diluted before adding 50μl per well and incubating
at 4°C for two hours. The plates were then washed with
25   0.1% NP-40 in PBS, before addition of 50 μl of detecting
polyclonal antisera at 1/1000 dilution. The plates were
washed again with 0.1% NP-40 in PBS and 50 μl of 1/1000
dilution peroxidase conjugated swine anti-rabbit Ig serum
(DAKO) was added for 2 h, then visualised by the TMB
30   reaction.

EXAMPLES

Example 1
Immunoprecipitation of MDM2, p53 and the MDM2-p53 complex
35   The observation that the rat cell line, Clone 6 expressed
a temperature sensitive mutant form of mouse p53 was
reexamined using a panel of p53 monoclonal antibodies.

21

Western blots were obtained of immunoprecipitates of MDM2, p53 and the MDM2-p53 complex from Clone 6 cells grown at 32°C for 24 hrs (Figure 1A) or continuously at 37°C (Figure 1B). The immunoprecipitates were obtained

5   using 1μg of purified antibody which were as follows: in lanes 1 and 4, - PAb421; in lanes 2 and 5, - Bp53-19; and in lanes 3 and 6, - 4B2. MDM2 was detected in lanes 1, 2 and 3 using SMP14 antibody supernatant and rabbit anti-mouse horse radish peroxidase conjugate; and p53 detected

10 in lanes 4, 5 and 6 using a 1 in 200 dilution of DM-1 and swine anti-rabbit horse radish peroxidase conjugate. An irrelevant antibody, PAb419, did not immunopecipitate either MDM2 or p53 from cell extracts prepared at either 32°C or 37°C (data not shown). The molecular weight of

15 the markers are given in kDa.

It was surprisingly found that one of the antibodies, Bp53-19, failed to immunoprecipitate p53 from Clone C6 cells grown at 32°C for 24 hours , but efficiently precipitated p53 from cells grown continuously at 37°C

20 (compare Figure 1A track 5 with Figure 1B track 5), whereas PAb421 precipitated p53 at both temperatures (Figure 1A track 4 and 1B track 4). Investigations were then carried out to determine whether Bp53-19 would co-immunoprecipitate MDM2 with p53. From the

25 immunoprecipitation western data in figure 1A and 1B it is clear that Bp53-19 does not co-immunoprecipitate MDM2 from cell extracts grown at 32 or 37°C (track 2 in figure 1A and B). Other p53 antibodies such as PAb421 do however co-immunoprecipitate MDM2 with p53 at 32°C but

30 not at 37°C (track 1 figure 1A and B). Conversely, antibodies against MDM2 such as 4B2, Figure 1, and SMP14 (data not shown) co-immunoprecipitate p53 at 32°C but not at 37°C (track 6 Figure 1A and B). The two bands recognized by 4B2 (and SMP14) at just below 80 kDa are

35 truncated forms of rat MDM2, as full length migrates on an SDS-PAGE gel with an apparent relative molecular mass

22

of 90kDa, multiple forms of MDM2 are often observed
(Chen, J., et al. (1993). Mol Cell Biol, 13, 4107-14).

Example 2

5   Two-site immunoassay to determine levels of MDM2, p53 and
MDM2-p53 complex
Two-site immunoassays were carried out to determine the
levels of MDM2, p53 and MDM2-p53 complex in Clone 6 cells
grown at 32°C for 24 hrs (Figure 2A) or continuously at

10   37°C (Figure 2B). In Figure 2A the coating antibodies
were one of the following purified antibodies as stated
in the figure legends: 4B2, 421 and Bp53-19, probed with
rabbit anti-p53 serum CM1 or rabbit anti-MDM2 serum, and
then detected using swine anti-rabbit horse radish

15   peroxidase conjugate and TMB as substrate. At 37°C the
MDM2-p53 complex was undetectable by any combination of
antibodies.

The two-site immunoassays of the levels of MDM2, p53 and

20   MDM2-p53 complex at 32°C and 37°C are consistent with the
immunoprecipitation results of Example 1. A striking
feature apparent from the data in figure 2A is that the
levels of p53 and p53-MDM2 complex are very similar
suggesting that most, but not all, p53 is in complex with

25   MDM2 at 32°C. The inability of Bp53-19 to detect a p53-
MDM2 complex at 32°C is again notable since other
combinations of antibodies are able to do so.

From comparison of the two-site immunoassays at 32°C and

30   37°C it is clear why MDM2 is not immunoprecipated at
37°C, as the levels of MDM2 protein are very much lower
and are only just detectable. No MDM2-p53 complex could
be detected by the two-site immunoassay of cell extracts
prepared at 37°C, see figure 2B, where the data for the

35   4B2 (as the capture antibody) and CM1 (as the detecting
antibody) combination of antibodies is shown (similarly
antibodies PAb421 or Bp53-19 and rabbit anti-MDM2

polyclonal did not detect the complex). The diminished
level of MDM2 at 37°C, less than 10% of that at 32°C, is
in contrast to the situation with p53 which is elevated
approximately 5 fold relative to the levels at 32°C.

5

The explanation for the ability of PAb421 and 4B2 only
being able to coprecipitate p53 and MDM2 together at
32°C, but not at 37°C is consistent with difference in
levels of MDM2 at the two temperatures, and also with the
10   published observations that mdm2 expression is dependent
on the "wild-type" form of p53 predominantly present at
32°C.

The failure of Bp53-19 to co-immunoprecipitate MDM2 or
15   detect the p53-MDM2 complex at 32°C is unexpected for two
reasons. Firstly, the two-site assay suggests there is
MDM2 protein in excess, which is able to form complexes
with p53 as detected by the capturing antibodies PAb421
and 4B2. Secondly, the two-site immunoassay at 37°C
20   suggests that Bp53-19 is almost as efficient as PAb421 at
recognizing p53 in the cell extracts. The simplest
interpretation for this observation is that Bp53-19
recognizes the same region on p53 that MDM2 binds to.

25

Example 3
Identification of MDM2-p53 binding site
It has previously been shown that Bp53-19 and MDM2
interact with the amino acid terminal end of p53 (Stephen
30   et al manuscript in preparation; Oliner, J.D., et al.
(1993). Nature, 362, 857-860). A complete peptide
library of the human p53 protein, and a partial peptide
library of the mouse p53 protein were available to
identify the region to which MDM2 binds. The human p53
35   sequence starts at peptide number 3 and ends at peptide
79, and each peptide consists of 15 amino acids, with the
last five amino acids being present in the next peptide

24

along. The mouse p53 sequence is partial and consists of
the N-terminal sequence from amino acid 1-92, again each
overlapping the next and previous peptide by five amino
acids.

5

These libraries consisted of 15 amino acid long sections
of the p53 primary amino acid sequence, that
consecutively overlapped by 5 amino acids, and were each
attached to biotin via a 4 amino acid long spacer. By

10   immobilizing the biotinylated peptides on streptavidin
coated ELISA plates the MDM2 binding site on p53 could be
quickly identified if it was encompassed within a stretch
of fifteen amino acids or less. Extract containing MDM2
was added to an ELISA plate with the peptide library

15   bound to it, and the bound MDM2 protein was later
detected using monoclonal antibody 4B2 and the standard
ELISA assay. Several sources of recombinant MDM2 protein
were used to challenge the p53 library, these included
crude extracts and partially purified preparations of

20   human and mouse MDM2 expressed in E.coli and also mouse
MDM2 expressed in insect cells; - all forms identified
the same peptides in the p53 library. The results using
the mouse MDM2 expressed in insect cells are shown in
figure 3A and B. The peptide library was challenged with

25   insect cell extract alone, SF9, and insect cell extract
expressing mouse MDM2, SF9 Mus MDM2. Binding of MDM2 to
the peptides was determined by an ELISA assay using
monoclonal antibody 4B2, and then detecting bound
antibody with rabbit anti-mouse Ig conjugated horse

30   radish peroxidase and TMB substrate. In Figure 3C is
shown the results from a control experiment using
peptides 59, 71, 83 and 95, as used in Figure 3B but
conducted in the presence or absence of extract to verify
the specificity of the detecting antibody, 4B2. The

35   results are presented alongside the ELISA readings for
extract of insect cells alone not expressing mouse MDM2.
The specificity is remarkable, - suggesting a strong

interaction between MDM2 and p53 derived peptides. From
the controls shown in figure 3C it can be seen that the
binding is only observed in the presence of extract
expressing MDM2, and is not due to the antibody
5   recognizing the peptide alone, moreover identical results
were obtained using SMP14 as the primary detecting
antibody (data not shown).

The four peptides that bind MDM2 are shown in figure 4.
10   Peptides 5 and 6 identify a site at the N-terminal end of
human p53, whereas peptides 83 and 84 identify the
corresponding region in the N-terminal end of mouse p53.
Collectively, these four peptides define the consensus
MDM2 binding site on p53 to be -QETFSD/GLWKL-,(SEQ ID
15   NOS:11 and 12) the aspartate to glycine being the only
amino acid difference between the human and mouse
sequence. The peptides involved in binding MDM2 are also
those recognized by the p53 antibodies DO-1 and Bp53-19
(Stephen et al, manuscript in preparation).

20

To define key residues on p53 that are involved in the
interaction with MDM2 a form of the consensus binding
site sequence -QETFSDLWKL- (SEQ ID NO:11) was modified by
substituting alanine at each position in the sequence and
25   determining what effect this had on the binding of MDM2
from the insect cell extract expressing MDM2. This
experiment was conducted in concert with examining the
effect on binding of the antibodies DO-1 and Bp53-19.
The results are presented in figure 5. The amino acid
30   sequences are as stated. In A the sequence
QETFSDLWKLLPENN (SEQ ID NO:1) represents the sequence of
peptide 6 from figure 3 and SPDDIEQWFTEDPGP (SEQ ID
NO:13) is an irrelevant peptide control. Formally the
first serine residue on the stated peptide is part of the
35   spacer coupling the consensus peptide to biotin, since
serin also precedes the consensus p53 sequence this
residue was also substituted with alanine. With regard

to MDM2 binding all alanine substitutions in the consensus binding site reduce the level of binding as measured by ELISA, however, the key residues would appear to be TFSDLW (SEQ ID NO:2) as substitutions in these positions reduce the amount of MDM2 binding to less than 15% of that seen with the unchanged consensus sequence. Interestingly, a higher level of binding of MDM2 is observed to the smaller consensus peptide rather than to peptide 6 (QETFSDLWKLLPENN) (SEQ ID NO:1) of the p53 peptide library reaffirming the definition of the binding site.   In the case of monoclonal antibody DO-1 binding to the consensus sequence the key residues are ETFSDLK, (SEQ ID NO:14) with D and K being the most crucial. The importance of the aspartate residue to the DO-1 epitope is consistent with the report that DO-1 only recognizes human p53 and not mouse p53, - the only difference being an aspartate to glycine change. While this difference has a critical affect on DO-1 binding it does not grossly affect the interaction of MDM2 Δ with the protein or peptides.   However substitution of alanine for aspartate at this position blocks binding of all three protein ligands. The ability of MDM2 to distinguish alanine from either glycine or aspartate at this position may imply that the polar environment of this region of the binding site is critical for the interaction. It has also been established from phage display libraries that the epitope of DO-1 is FSDLWKL (SEQ ID NO:15) (Stephen et al, manuscript in preparation), which is in agreement with our observations on key residues. For the antibody Bp53-19 the alanine substitution series identifies the key residues to be F-DLW- (SEQ ID NO:16) with the latter three residues being the most crucial, and is similar to the requirements for MDM2 binding to the consensus binding site. Not surprisingly, it was found that the pre-binding of antibody Bp53-19 onto he SQETFSDLWKL (SEQ ID NO:17) biotinylated peptide blocked binding of MDM2 to the peptide when added later (data not shown).

5

10

15

20

25

30

35

Example 4

Further characterisation of the p53-MDM2 binding site

The process of Example 3 was repeated but using insect
cells infected with baculovirus expressing Mus MDM2 from
5   SF9 cells in two 180ml^2 tissue culture flasks. The
extract was prepared in approximately three mils of
lysisi buffer to give a concentrated supernatant of 13
mg/ml.

10   As before, the MDM2 binding site was defined by alanine
substitution of the p53 derived peptide, SQETFSDLWL (SEQ
ID NO:18) and the results are shown in Figure 6.
Additionally other conserved substitutions were tested
(i.e those commonly seen in highly conserved proteins of
15   identical function) and the results are also shown in
Figure 6. With a higher protein concentration, (13 mg/ml
as opposed to 1-4 mg/ml) the alanine substitution
experiment reveals the same six amino acids are important
(^{18}TFSDLW^{23})(SEQ ID NO:2) in addition to glutamate
20   (ETFSDLW)(SEQ ID NO:19). This data however establishes
that the most critical residues are F--LW (SEQ ID NO:4)
as these are intolerant of both alanine substitutions and
some, if not all of the conserved substitutions.

25   A further interesting observation relates to the fact
that substitution of the aspartate residue for the
glutamate enhances binding of MDM2, indicating that such
a residue may usefully be included in the therapeutic
peptides of the invention. This also illustrates that
30   this approach can lead to the discovery of agents that
have enhanced binding to MDM2.

Additional References

Dyson, N., et al. (1992b). J. Virol., 66, 6893-6902.

Houghten, R.A., et al. (1991). Nature, 354, 84-86.

5   Lu, X., et al (1992). Cell, 70, 153-161.

CLAIMS

5
1.   A compound for use in interfering with the binding
between p53 and MDM2 or a protein having a p53 binding
site analogous to that of MDM2, which compound comprises
a peptide having up to twenty eight amino acids which is
able to disrupt or prevent binding between p53 and MDM2,
or a functional peptide analogue thereof.

10
2.   The compound of claim 1 which comprises a peptide
having from 4 to 10 amino acids.

3.   The compound of claim 1 or claim 2 which comprises a
peptide which comprises a fragment of p53 protein which
15
includes at least some of amino acids 18-23 within the
sequence of human p53, or a peptide analogue thereof.

4.   The compound any one of the preceding claims which
comprise a peptide comprising the sequence FxxLW (SEQ ID
20
NO:4).

5.   The compound of claim 4 wherein the peptide
comprises the sequence FxELW (SEQ ID NO:5).

25
6.   The compound of claim 4 or claim 5 wherein the
peptide comprises the sequence TFSDLW (SEQ ID NO:2).

7.   The compound of any one of the preceding claim
wherein the oncogene protein is human MDM2.
30
8.   The compound of claim 1 which comprises the compound
an organic compound which is modelled to resemble the
three dimensional structure of the amino acid residues
F LW (SEQ ID NO:4) as they appear at amino acid residues
35
19-23 in human p53 and which binds to human MDM2.

9.   A method of identifying compounds which interfere

with the binding of oncogene protein to p53 which
comprises exposing a fragment of p53 protein which
includes at least some of amino acids 18-23 within the
sequence of human p53, or a peptide analogue thereof, to

5   the oncogene protein or a binding fragment thereof in the
presence of a compound under test, and detecting bound
complex of either the fragment of p53 or peptide
analogue, and/or the compound under test with the
oncogene protein or binding fragment.

10

10.   A method of identifying compounds which interfere
with the binding of human MDM2 to human p53, said method
comprising
       immobilising a first peptide molecule,

15   adding a compound to be tested and a second peptide
molecule; detecting the presence of bound second peptide
at the immobilisation site;
wherein one of the first peptide or second peptide is
MDM2 or an oncogene protein having a p53 binding site

20   analogous to that of MDM2 or a fragment thereof which
includes said binding site, and the other is a fragment
of human p53 of from five to twenty eight amino acids
including the amino acid residues FxxLW (SEQ ID NO:4) or
a peptide analogue thereof.

25

11.   The method of claim 9 or claim 10 wherein the said
fragment of p53 or peptide comprises a fragment of human
p53 of from 12 to 28 amino acids including the sequence
FxxLW (SEQ ID NO:4).

30

12.   The method of any one of claims 9 to 11 wherein acid
peptide analogue is a peptide which includes sequence
FxxLW (SEQ ID NO:4).

35   13.   The method of any one of claims 9 to 12 which is
adapted to provide a high throughput screen.

14.   The method of any one of claims 9 to 13 wherein the
compounds are obtained from synthetic combinatorial
peptide libraries.

5   15.   The method of any one of claims 9 to 14 wherein the
said oncogene protein is human MDM2.

16.   A method of identifying compounds which interfere
with the binding of human MDM2 to human p53, said method
10   comprising binding a predetermined quantity of a first
peptide which is detectably labelled to a peptide, adding
a compound to be tested; and determining the quantity of
the first protein which is displaced from or prevented
from binding to the second peptide, wherein one of the
15   first peptide or the second peptide is MDM2 or a peptide
having a p53 binding site analogous to that of MDM2, and
the other is a fragment of human p53 of from six to
twenty eight amino acids including the amino acid
residues 18-23 in the sequence of human p53 as set out in
20   WO93/20238, or a peptide analogue thereof.

17.   A compound for use in interfering with the binding
of p53 and MDM2 a method of claim 1 which is identified
by a method according to any one of claims 9 to 16.

25

18.   A novel compound identified by the method of any one
of claims 9 to 18.

19.   A novel compound selected from the group comprising
30   a peptide of from four to twenty eight amino acids which
is able to disrupt or prevent the binding between p53 and
an MDM2, or a peptide analogue thereof.

20.   The compound of claim 19 which comprises a
35   polypeptide of from six to ten amino acids in length.

21.   A polypeptide which interferes with the binding of

32

human MDM2 to human p53, which compound comprises a
polypeptide of from five to twenty eight amino acids in
length, said polypeptide being coincidental with or a
variant of a portion of p53 protein, said portion
5   including amino acids F--LW (SEQ ID NO:4) as contained
within amino acids 18-23 within the sequence of human p53
as set out in WO93/20238.

22. A polypeptide which binds with human MDM2, which
10   compound comprises a polypeptide of from five to twenty
eight amino acids in length, said polypeptide comprising
a portion of p53 protein, said portion including the
amino acid sequence TFSDLW (SEQ ID NO:2).

15   23. A compound which is modelled to resemble the three
dimensional structure of the sequence F--LW (SEQ ID NO:4)
as it appears in human p53 and which binds to human MDM2.

20   24. A compound for use in inhibiting the growth of
tumour cells which contain a human MDM2 gene
amplification, which compound interferes with the binding
between p53 and an MDM2 and comprises a peptide having up
to twenty eight amino acids which is able to disrupt or
25   prevent the binding between p53 and MDM2, or a functional
peptide analogue thereof.

25. A DNA molecule for use in inhibiting the growth of
tumour cells which contain a human MDM2 gene
30   amplification, said DNA molecule encoding a polypeptide
comprising a portion of p53 or a variant thereof, said
portion comprising amino acids 18-23 of p53, said
polypeptide being capable of binding to human MDM2.

35   26. A pharmaceutical composition comprising an effective
amount of a compound selected from the group consisting
of a peptide having up to twenty eight amino acids which

is able to disrupt or prevent binding between p53 and
MDM2 or a peptide analogue thereof in combination with
pharmaceutically acceptable carrier or diluent.

5   27.   The composition of claim 21 which further comprises
a different anti-tumour chemotherapeutic agent.

28.   A diagnostic method for detecting the presence of
MDM2 comprising applying to a sample under test a
10   fragment of p53 protein which includes at least some of
amino acids 18-23 within the sequence of human p53, or a
peptide analogue thereof, and detecting the presence of
bound complex using antibodies specific for MDM2.

15   29.   An assay kit comprising a a fragment of p53 protein
which includes at least some of amino acids 18-23 within
the sequence of human p53, or a peptide analogue thereof,
and at least one antibody capable of detecting the
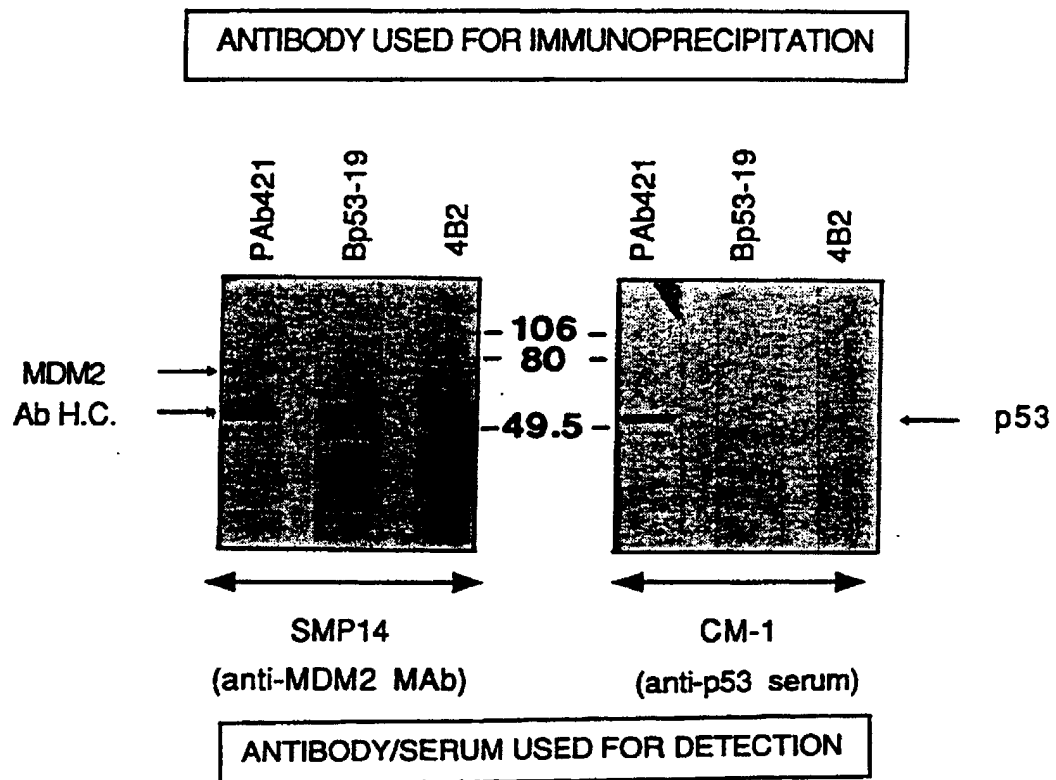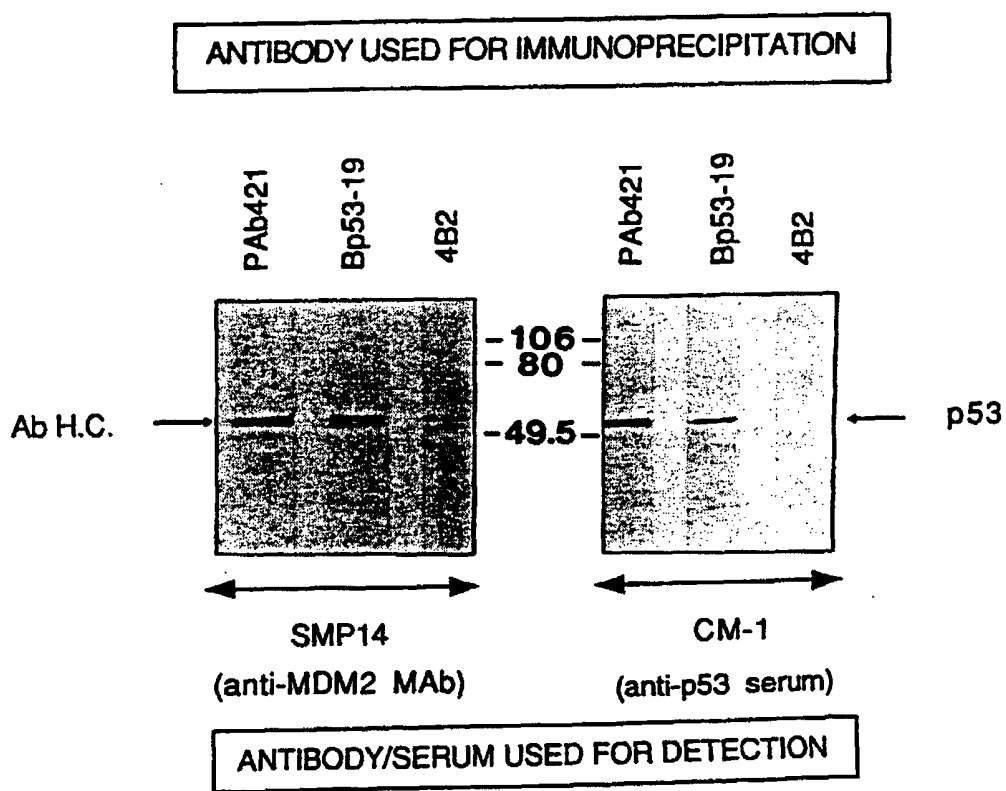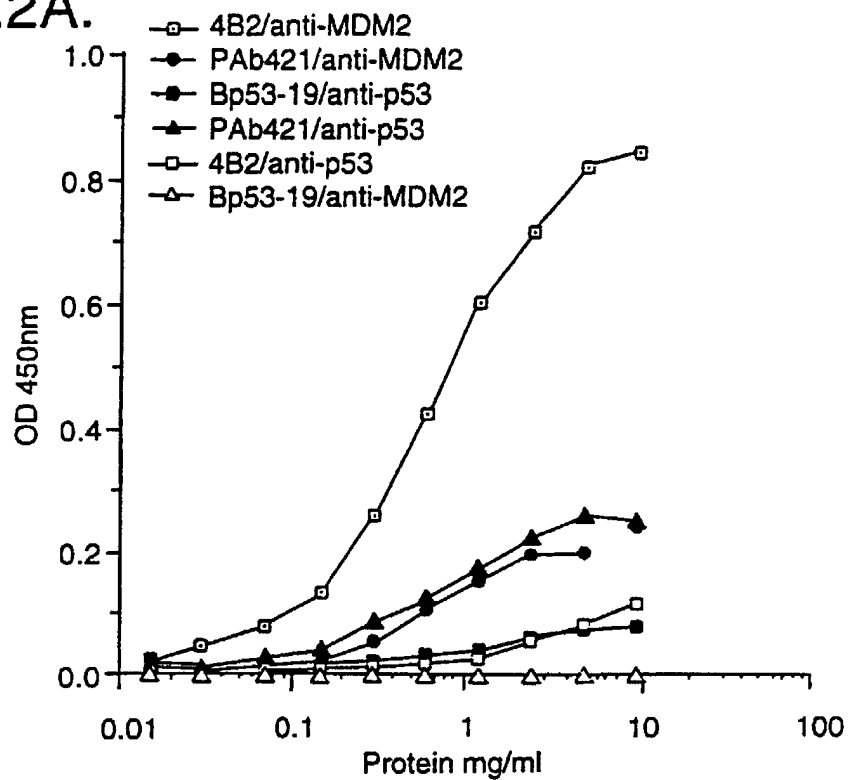presence of bound MDM2.

20

Fig.1A.

ANTIBODY USED FOR IMMUNOPRECIPITATION

SMP14
(anti-MDM2 MAb)

CM-1
(anti-p53 serum)

ANTIBODY/SERUM USED FOR DETECTION

Fig.1B.

ANTIBODY USED FOR IMMUNOPRECIPITATION

ANTIBODY/SERUM USED FOR DETECTION

Fig.2A.

Legend:
- □ 4B2/anti-MDM2
- ● PAb421/anti-MDM2
- ■ Bp53-19/anti-p53
- ▲ PAb421/anti-p53
- □ 4B2/anti-p53
- △ Bp53-19/anti-MDM2

Y-axis: OD 450nm (0.0 to 1.0)
X-axis: Protein mg/ml (0.01 to 100)

Fig.2B.

MAb / Polyclonal
- □ 4B2/anti-MDM2
- ■ Bp53-19/anti-p53
- ▲ 421/anti-p53
- △ 4B2/anti-p53
and Bp53-19/anti-MDM2
and PAb421/anti-MDM2

Y-axis: OD 450nm (0.0 to 1.0)
X-axis: Protein mg/ml (0.01 to 100)

Fig.3A.

Fig.3B.

Legend:
- □ Sf9
- ▨ Sf9/Mus MDM2

Y-axis: OD 450nm (0.0 to 0.7)

X-axis: Peptide number (51 to 96)

Fig.3C.

Fig.4.

```
        11                    25
#5     -EPPLSQETFSDLWKL-
                                          ⎤
                                          ⎥
        16                       30       ⎥  HUMAN p53 SEQUENCE
#6        -QETFSDLWKLLPENN-               ⎥
                                          ⎦


        13                    27
#83    -PLSQETFSGLWKLLP-
                                          ⎤
                                          ⎥
          18                   32         ⎥  MOUSE p53 SEQUENCE
#84       -TFSGLWKLLPPEDIL-               ⎥
                                          ⎦

CONSENSUS MDM2      -QETFSD/GLWKL-
BINDING SITE
```
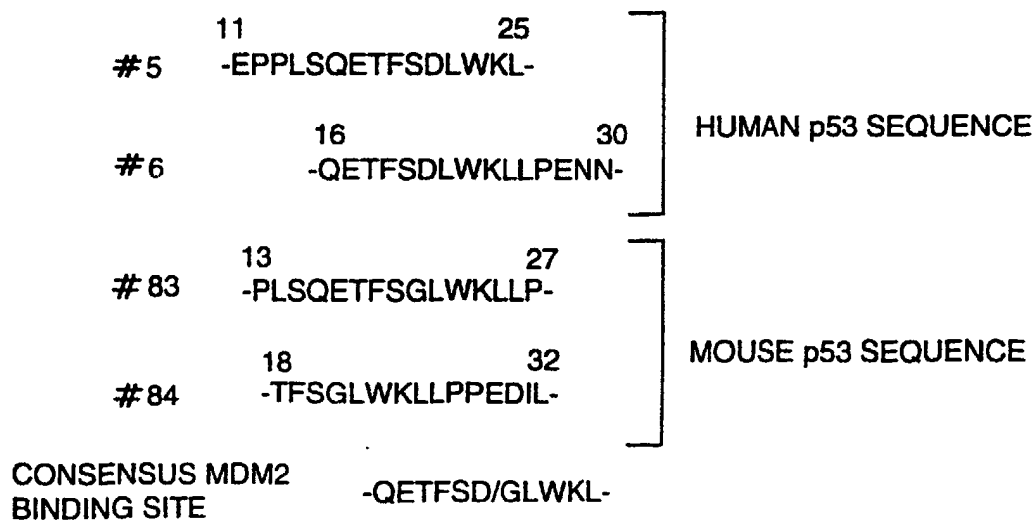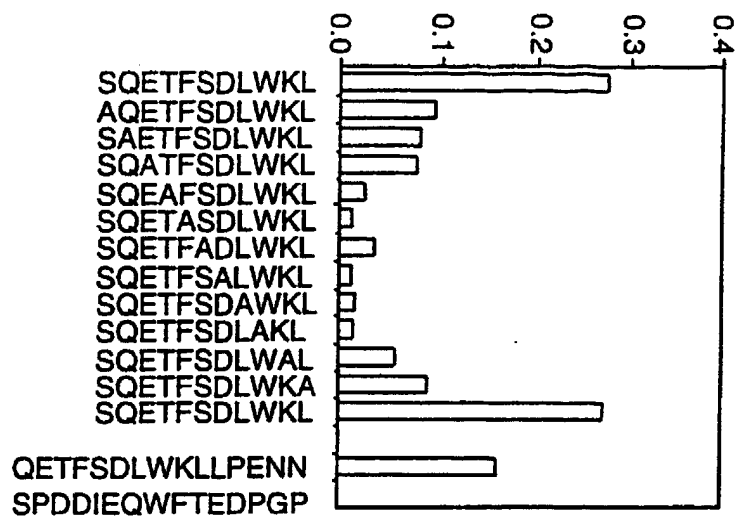
OD 450nm

Fig.5A.

Fig.5B.

Fig.5C.

Fig.6.

Further information on the key residues required for MDM2 binding
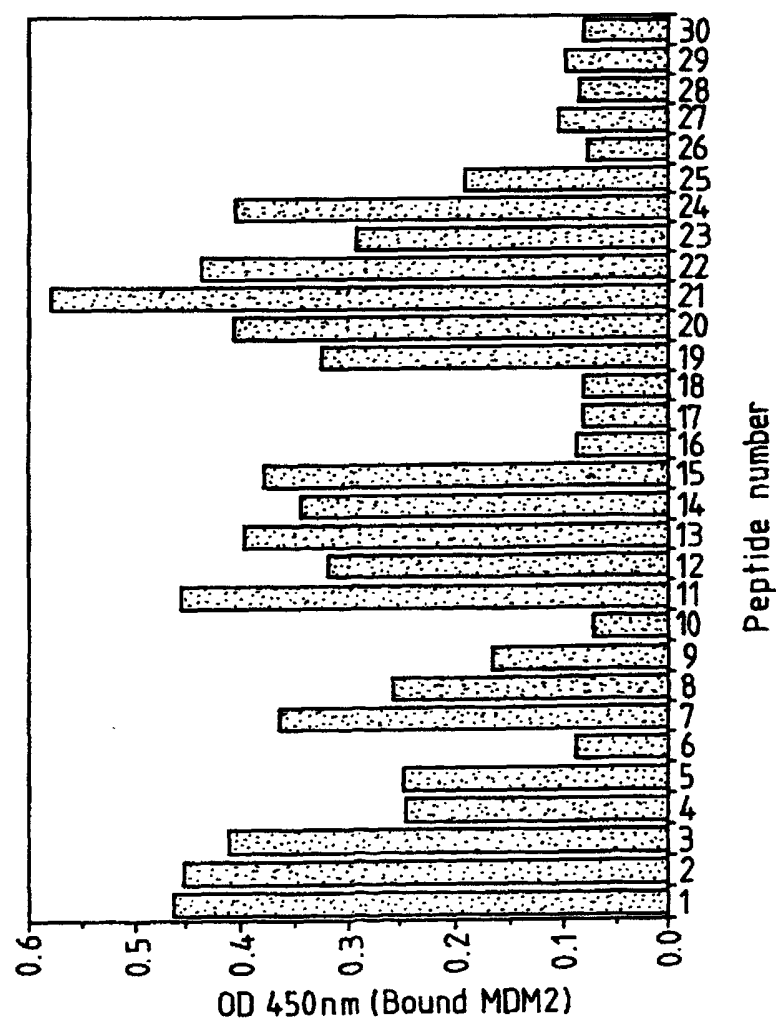
NO.	PEPTIDE	CHANGE
1.	SQETFSDLWKL	-
2.	AQETFSDLWKL	S TO A
3.	SAETFSDLWKL	Q TO A
4.	SQATFSDLWKL	E TO A
5.	SQEAFSDLWKL	T TO A
6.	SQETASDLWKL	F TO A
7.	SQETFADLWKL	S TO A
8.	SQETFSALWKL	D TO A
9.	SQETFSDAWKL	L TO A
10.	SQETFSDLAKL	W TO A
11.	SQETFSDLWAL	K TO A
12.	SQETFSDLWKA	L TO A
13.	DQETFSDLWKL	S TO D
14.	SQETFDDLWKL	S TO D
15.	SQESFSDLWKL	T TO S
16.	SQETISDLWKL	F TO I
17.	SQETLSDLWKL	F TO L
18.	SQETMSDLWKL	F TO M
19.	SQETFTDLWKL	S TO T
20.	SQETFPDLWKL	S TO P
21.	SQETFSELWKL	D TO E
22.	SQETFSQLWKL	D TO Q
23.	SQETFSNLWKL	D TO N
24.	SQETFSDMWKL	L TO M
25.	SQETFSDIWKL	L TO I
26.	SQETFSDLRKL	W TO R
27.	SQETFSDLEKL	W TO F
28.	SQETFSDLYKL	W TO Y
29.	SQETFSFLIKL	TAg core seq.
30.	TAg first 15aa	

Fig.7.

Biotinylated peptide containing MDM2 binding
site of p53 added to streptavidin-coated plate

Add MDM2 (peptide or polypeptide) in solution + or – test agents

No agent   + test agent 1   + test agent 2
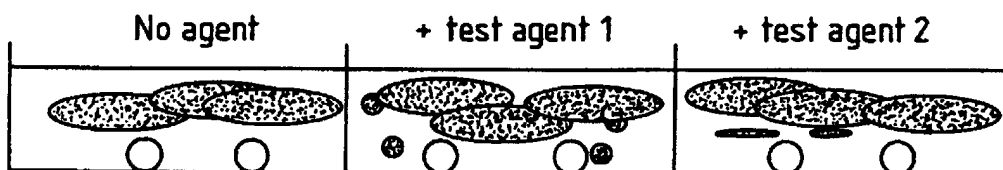
Wash away unbound MDM2. Determine bound MDM2 by ELISA

ELISA
reading   ++   +   +++

(reference)   (below reference)   (above reference)
                      Test agent disrupts   Test agent enhances
                      p53-MDM2 interaction   p53-MDM2 interaction

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/12   C07K14/82   C07K14/47   G01N33/574   A61K38/17
A61K38/04

According to International Patent Classification (IPC) or to both natio: classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12N   C07K   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searc: :

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 20238 (THE JOHN HOPKINS UNIVERSITY) 14 October 1993 cited in the application see page 11 see page 19 last paragraph see page 24 - page 26; example 7 see figure 6 see claims 30, 33, 41	1-19, 21-26, 28,29
X	MOL. CELL. BIOL., vol. 13, no. 7, July 1993 pages 4107-4114, CHEN ET AL. 'Mapping of the p53 and mdm-2 interaction domains' see page 4112	25
Y		1-24, 26-29

-/--

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 December 1995	05. 01. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gac, G

2

Form PCT/ISA/210 (second sheet) (July 1992)

Internatic   Application No

PCT/GB 95/01719

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GENES DEV., vol. 8, no. 10, 15 May 1994 pages 1235-1246, LIN ET AL. 'Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-KD protein' see the whole document ---	1-24, 26-29
X	WO,A,94 08241 (DUETSCHES KREBSFORSCHUNGSZENTRUM STIFTUNG DES ÖFFENTLICHEN RECHTS) 14 April 1994 see page 3 third paragraph see page 19 fragment 8 seepage 25 fragment 8 ---	1-6, 18-23
X	MOL. CELL. BIOL., vol. 12, no. 11, November 1992 pages 5051-5049, LEES-MILLER ET AL. 'Human DNA-activated protein-kinase phosphorylates serines 15 and 37 in the amino-terminal transactivation domain of human p53' see table 1 ---	1-8, 17-24
P,X	ONCOGENE, vol. 9, no. 9, September 1994 pages 2523-2529, PICKSLEY ET AL. 'Immunochemical analysis of the interaction of p53 with mdm2 ; fine mapping of the mdm2-binding site on p53 using synthetic peptides' see the whole document ---	1-29
X	WO,A,94 10306 (LABORATOIRES EUROBIO) 11 May 1994 see the whole document ---	1-6, 17-23
A	WO,A,94 00601 (THE TRUSTEES OF PRINCETON UNIVERSITY) 6 January 1994 see the whole document -----	1-29

2

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9320238	14-10-93	US-A-	5411860	02-05-95
		AU-B-	4278893	08-11-93
		CA-A-	2133306	14-10-93
		EP-A-	0635068	25-01-95
		JP-T-	7505294	15-06-95
		US-A-	5420263	30-05-95
WO-A-9408241	14-04-94	EP-A-	0614531	14-09-94
		JP-T-	7501711	23-02-95
WO-A-9410306	11-05-94	FR-A-	2698367	27-05-94
		AU-B-	5424694	24-05-94
		CA-A-	2148274	11-05-94
		EP-A-	0666913	16-08-95
WO-A-9400601	06-01-94	AU-B-	4544393	24-01-94
		AU-B-	4545193	24-01-94
		CA-A-	2137822	06-01-94
		EP-A-	0654092	24-05-95
		WO-A-	9400603	06-01-94